Summary of ACMG-AMP Criteria for AKT3, MTOR, PIK3CA and PIK3R2

Gene	Disease (MONDO ID)	Clinically significant transcript
AKT3	**MONDO:0016054**	NM_005465.4
MTOR	**MONDO:0016054**	NM_004958.3
PIK3CA	**MONDO:0016054**	NM_006218.3
PIK3R2	**MONDO:0016054**	NM_005027.3

Table 1

PATHOGENIC CRITERIA		
Criteria	**Criteria Description**	**Specification**
VERY STRONG CRITERIA		
PVS1	Null variant in a gene where loss of function is a known mechanism of disease.	N/A
PS4_Very Strong	The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls. *Points are assigned for phenotype according to (**Supplementary Table 3**). Strength of evidence is determined by points according to (**Supplementary Table 2**).* *PS4_VeryStrong = >16 points*	Disease-specific; Strength
STRONG CRITERIA		
PS1	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change.	None
PS2	*De novo* (paternity confirmed) in a patient with the disease and no family history. *Award the PS2_Moderate point if Criteria 1 is fulfilled, OR if parents are not available but Criteria 2 is fulfilled. Award the PS2_Strong point if Criteria 1 AND Criteria 2 are fulfilled. Note: Only a moderate point can be awarded.*	Disease-specific; Strength

PS3	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect *Follow recommendations set forth by the SVI in conjunction with specifications added by the Brain Malformation Group for quality metrics and minimum validation controls required which do not apply to animal models. Animal model generated with the variant of interest expressed in neural progenitors show a complementary brain phenotype.* (**Supplementary Document 1**)	Disease-specific
PS4	The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls. *Points are assigned for phenotype according to (**Supplementary Table 3**). Strength of evidence is determined by points according to (**Supplementary Table 2**).* *PS4 = 3.5-15.99 points*	Disease-specific
MODERATE CRITERIA		
PM3	For recessive disorders, detected in trans with a pathogenic variant.	N/A
PM4	Protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants.	N/A
PM5	Missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before.	None
PM6	Confirmed de novo without confirmation of paternity and maternity. Addressed by PS2	N/A
PS3_Moderate	Follow recommendations set forth by the SVI in conjunction with specifications added by the Brain Malformation Group for quality metrics and minimum validation controls required which do not apply to animal models. Animal model generated with the variant of interest expressed in non-neural tissues show an increased cancer burden. (**Supplementary Document 1**)	Strength
PS4_Moderate	The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls. *Points are assigned for phenotype according to (**Supplementary Table 3**). Strength of evidence is determined by points according to (**Supplementary Table 2**).*	Strength

	PS4_Moderate = 1.5-3.49 points	
SUPPORTING CRITERIA		
PP1	Co-segregation with disease in multiple affected family members Not applicable since disease-causing variants are germline mosaic, *de novo* or mosaic.	N/A
PP2	Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease. Constraint metrics as computed in ExAC/gnomAD with the cut-off z-score > 3.09 which is applicable to *MTOR, PIK3CA and AKT3* but not *PIK3R2*	Disease-specific
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or gene product	N/A
PP4	Phenotype specific for disease with single genetic etiology. This criterion is accounted for under PS4.	N/A
PP5	*Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation*	
PM1_Supporting	Located in a mutational hot spot and/or critical and well-established functional domain. *Applicable for each gene; see Table 4 for regions by gene*	Strength
PM2_Supporting	Absent/rare from controls in an ethnically-matched cohort population sample.	Disease-specific
PS3_Supporting	Follow recommendations set forth by the SVI in conjunction with specifications added by the Brain Malformation Group for quality metrics and minimum validation controls required (**Supplementary Document 1**)	Strength
PS4_Supporting	The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls. *Points are assigned for phenotype according to (**Supplementary Table 3**). Strength of evidence is determined by points according to (**Supplementary Table 2**).* *PS4_Supporting = 0.5 - 1.49 points*	Strength

BENIGN CRITERIA		
Criteria	**Criteria Description**	**Specification**
STAND ALONE CRITERIA		
BA1	Allele frequency above ≥0.185%	Disease-specific
STRONG CRITERIA		
BS1	Allele frequency greater than expected for disease (≥0.037%)	Disease-specific
BS2	Observed in the homozygous state in a healthy adult *Clinical laboratories are encouraged to accumulate ≥3 instances of well phenotyped family members before applying this strong criterion*	Disease-specific
BS3	Well-established in vitro or in vivo functional studies shows no damaging effect on protein function *Follow recommendations set forth by the SVI in conjunction with specifications added by the Brain Malformation Group for quality metrics and minimum validation controls required (**Supplementary Document 1**)*	Disease-specific
BS4	Lack of segregation in affected members of a family.	N/A
SUPPORTING CRITERIA		
BP1	Missense variant in gene where only LOF causes disease	N/A
BP2	Observed in trans with a pathogenic variant for a fully penetrant dominant gene/disorder; or observed in cis with a pathogenic variant in any inheritance pattern.	None
BP3	In-frame deletions/insertions in a repetitive region without a known function	N/A
BP4	Multiple lines of computational evidence suggest no impact on gene or gene product	Disease-specific
BP5	Variant found in a case with an alternate molecular basis for disease	None

BP6	*Reputable source recently reports variant as benign but the evidence is not available to the laboratory to perform an independent evaluation*	N/A
BP7	A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved. For synonymous, intronic positions (except canonical splice sites) and non-coding variants in the UTRs, if the nucleotide is non-conserved award this point	Disease-specific
BS3_Supporting	Follow recommendations set forth by the SVI in conjunction with specifications added by the Brain Malformation Group for quality metrics and minimum validation controls required (**Supplementary Document 1**)	Strength

Key: **Disease-Specific:** Disease-specific modifications based on what is known about MTORopathies; **Strength:** Increasing or decreasing strength of criteria based on the amount of evidence; **N/A:** not applicable; **None:** no changes made to existing criteria definitions.

VERY STRONG EVIDENCE OF PATHOGENICITY

PVS1	Null variant (nonsense, frameshift, canonical +/-1 or 2 splice sites, initiation codon, single or multi-exon deletion) in a gene where loss of function (LOF) is a known mechanism of disease Caveats: • Use caution interpreting LOF variants at the extreme 3' end of a gene • Use caution with splice variants that are predicted to lead to exon skipping but leave the remainder of the protein intact Brain Malformation Recommendation: LOF and/or haploinsufficiency have not been clearly identified as disease mechanisms underlying brain malformations related to these genes, so in general this rule is not applicable. The disease mechanism for these genes is gain of function (GOF).

PS4_VeryStrong	The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls. Brain Malformation Recommendation: For PS4, for cases reported in the literature, we recommend assigning each one to the **SINGLE** category below that is associated with the highest point value (Supplementary Table 3).* The total score obtained for all reported cases with a particular variant will determine the strength of PS4 assigned according to the scale (Supplementary Table 2).** PS4_VeryStrong = >16 points ***Applicable if the variant is absent from controls according to PM2 to ensure the variant is not simply present due to beinging common in the general population*

STRONG EVIDENCE OF PATHOGENICITY

PS1	Same amino acid change as a previously established pathogenic variant regardless of nucleotide change Brain Malformation Recommendation: Previously established variant must meet criteria as pathogenic per Brain Malformation Expert Panel Criteria Independent of this point.
PS2	*De novo* (both maternity and paternity confirmed) in a patient with the disease and no family history Note: Confirmation of paternity only is insufficient. Egg donation, surrogate motherhood, errors in embryo transfer, *etc.* can contribute to non-maternity Brain Malformation Recommendation: Award the PS2_Moderate point if Criteria 1 is fulfilled, OR if parents are not available but Criteria 2 is fulfilled. Award the PS2_Strong point if Criteria 1 AND Criteria 2 are fulfilled. Note: Only a moderate point can be awarded. Criteria 1. The variant is present at a detectable allele fraction but is absent from parental samples with confirmed maternity and paternity. Criteria 2. The variant is present at a detectable allele fraction in an affected tissue sample but is absent from or detected at a lower allelic fraction in another tissue (e.g. if present in 5% of brain tissue but absent from the blood or skin this point can be awarded*) *†For the sake of implementation, these criteria are intended to apply to high-confidence somatic mutations identified by the reporting CLIA laboratory. The expert panel recognizes that in practice there may be significant heterogeneity in the technical methods and thresholds used to identify such variants as "high confidence", and flags the need to establish consensus statistical frameworks (e.g. Phred-scaled genotype qualities) or experimental approaches (e.g., confirmation of somatic variants by sequencing on orthogonal platforms) by which quality thresholds can be consistently applied.*

PS3	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or gene product Brain Malformation Recommendation: Award PS3 if the functional assay meets the acceptability criteria delimited in PMID: 31892348 and Supplementary Document 1. These assays are typically conducted on a research basis and not by CLIA laboratories. Note: Phosphorylation levels in human cells can be very variable and can be influenced by various biological fluctuations, including insulin state, nutrition/starvation state, cell type, etc., and there does not yet exist sufficient evidence to establish an absolute standard reference range. Therefore, awarding this point requires an experienced reader to assess the experimental literature according to the criteria outlined below. This includes ensuring that appropriate positive and negative controls have been utilized. Caveat: Studies of cell lines derived from the affected patient as the only source of functional characterization are by themselves insufficient to provide strong evidence of pathogenicity. This is because cells derived from patient affected tissue are likely to exhibit the desired phenotype since the patient tissue exhibits the phenotype. It is therefore impossible to determine whether the variant of interest was solely responsible for that phenotype. Instead, functional readout of patient derived cells are now included in PS4.
PS4_Strong	The prevalence of the variant in affected individuals is significantly increased compared to the prevalence in controls Brain Malformation Recommendation: PS4_Strong = 3.5-15.99 points

MODERATE EVIDENCE OF PATHOGENICITY

PM1	Located in a mutational hot spot and/or critical and well-established functional domain (*e.g.* active site of an enzyme) without benign variation Brain Malformation Recommendation: Not applicable since the strength of this point has been modified
PM3	For recessive disorders, detected in *trans* with a pathogenic variant Note: This requires testing of parents (or offspring) to determine phase Brain Malformation Recommendation: Not applicable since disease-causing variants are heterozygous
PM4	Protein length changes due to in-frame deletions/insertions in a non-repeat region or stop-loss variants Brain Malformation Recommendation: Although there have been reported in-frame deletion/insertions in these genes which cause the overgrowth phenotype, they are exceptionally rare. Most insertion/deletions are associated with a LoF disease mechanism and so this point will still not be used even though we recognize that it is possible that a variant is an in-frame indel that results in a GoF mechanism.

PM5	Missense change at an amino acid residue where a different missense change determined to be pathogenic has been seen before Brain Malformation Recommendation: Previously established variant must meet criteria as pathogenic per Brain Malformation Expert Panel Criteria Independent of this point.
PM6	Assumed *de novo*, but without confirmation of paternity and maternity Brain Malformation Recommendation: This point is addressed according to PS2 and will not be used.
PS3_Moderate	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or gene product Brain Malformation Recommendation: Follow recommendations set forth by the SVI in conjunction with specifications added by the Brain Malformation Group for quality metrics and minimum validation controls required PMID: 31892348 (**Supplementary Document 1**)
PS4_Moderate	The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls. Brain Malformation Recommendation: PS4_Moderate = 1.5-3.49 points

SUPPORTING EVIDENCE OF PATHOGENICITY

PP1	Co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease Brain Malformation Recommendation: Not applicable since disease-causing variants are germline mosaic, *de novo* or mosaic.
PP2	Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease Brain Malformation Recommendation: We will use constraint metrics as computed in ExAC with the cut-off z-score > 3.09 which is applicable to all four genes described and curated herein
PP3	Multiple lines of computational evidence support a deleterious effect on the gene or gene product (conservation, evolutionary, splicing impact, etc) Brain Malformation Recommendation: This criterion is not applicable since these variants are GOF, and traditional mutation pathogenicity prediction algorithms focus on LOF mechanisms. Use of this criterion can be revisited if there emerges additional

	published experience with predictive algorithms specifically designed to detect gain of function mutations.
PP4	Patient's phenotype or family history is highly specific for a disease with a single genetic etiology. Brain Malformation Recommendation: Not applicable since this criterion is accounted for under PS4.
PP5	Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation Not Applicable
PM1_Supporting	Located in a mutational hot spot and/or critical and well-established functional domain (*e.g.* active site of an enzyme) without benign variation Brain Malformation Recommendation: A supporting point may be awarded if the variant of interest is located in an exon within one of the below approved functional domains Supplemental table 4. Please note that specific residues subject to recurrent gain-of-function mutations are not covered by this criterion; these are instead accounted for by PS4.
PM2 _Supporting	Absent from controls (or at extremely low frequency if recessive) in Exome Sequencing Project, 1000 Genomes or ExAC/gnomAD Brain Malformation Recommendation:(One person maximum) This criterion accounts for sequencing artifacts that may have been included in population databases. This number accounts for false calls due to sequencing/ calling errors since the data sets from ExAC/gnomAD are from various sources and GATK calling is also known to call false positives. (PMID: 22827831) This criteria as been downgraded to supporting per recommendation by the SVI working group.
PS3_Supporting	Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or gene product Brain Malformation Recommendation: Follow recommendations set forth by the SVI in conjunction with specifications added by the Brain Malformation Group for quality metrics and minimum validation controls required PMID: 31892348 (**Supplementary Document 1**)
PS4_Supporting	The prevalence of the variant in affected individuals is significantly increased compared with the prevalence in controls. Brain Malformation Recommendation: PS4_Supporting = .5-1.49 points

STAND ALONE EVIDENCE OF BENIGN IMPACT

BA1	Allele frequency is above 5% in Exome Sequencing Project, 1000 Genomes, or ExAC Brain Malformation Recommendation:An allele frequency ≥0.185% was approved *Note: this was adjusted from ACMG Guidelines due to maintaining the 5x threshold for benign (consistent with previously established guidelines)*

STRONG EVIDENCE OF BENIGN IMPACT

BS1	Allele frequency is greater than expected for disorder Brain Malformation Recommendation: An allele frequency ≥0.037% was approved. (Supplemental Table 5)
BS2	Observed in a healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder with full penetrance expected at an early age. Brain Malformation Recommendation: Clinical laboratories are encouraged to accumulate more than 2 (≥3) instances of well phenotyped family members before applying this strong criterion. To be considered for this point, the variant should be either germline (most common), or somatic in a relevant tissue. Homozygous occurrences in gnomAD or ExAC can also be counted for this point (≥3)
BS3	Well-established in vitro or in vivo functional studies shows no damaging effect on protein function. Brain Malformation Recommendation: Follow recommendations set forth by the SVI in conjunction with specifications added by the Brain Malformation Group for quality metrics and minimum validation controls required PMID: 31892348 (**Supplementary Document 1**)
BS4	Lack of segregation in affected members of a family Brain Malformation Recommendation: Not applicable as these are *de novo*, germline mosaic or post-zygotic mutations.

SUPPORTING EVIDENCE FOR BENIGN IMPACT

BP1	Missense variant in a gene for which primarily truncating variants are known to cause disease Brain Malformation Recommendation: Not applicable as LOF is not the disease mechanism
BP2	Observed in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder; or observed in *cis* with a pathogenic variant in any inheritance pattern

	Brain Malformation Recommendation: Observed in *cis* or *trans* with a known pathogenic variant in the same gene
BP3	In-frame deletions/insertions in a repetitive region without a known function Brain Malformation Recommendation: This is not applicable for the genes specified since the exon regions do not have repetitive regions without a known function.
BP4	Multiple lines of computational evidence suggest no impact on gene or gene product (conservation, evolutionary, splicing impact, etc.) Brain Malformation Recommendation: Not applicable for any variant type except for synonymous and intronic variants. This criterion can be applied when two of three splicing prediction tools predict no splicing change. The splicing prediction tools used are: varSEAK, spliceAI and MaxEntScan.
BP5	Variant found in a case with an alternate molecular basis for disease Brain Malformation Recommendation: Observed in a case with an alternate molecular basis for disease in a different gene
BP6	Reputable source recently reports variant as benign but the evidence is not available to the laboratory to perform an independent evaluation Brain Malformation Recommendation: Not applicable
BP7	A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved Brain Malformation Recommendation: For synonymous, intronic positions (except canonical splice sites) and non-coding variants in the UTRs, if the nucleotide is non-conserved award this point. Not conserved is defined as the same nucleotide NOT present in all vertebrae or PhyloP score <0.1.
BS3_Supporting	Well-established in vitro or in vivo functional studies shows no damaging effect on protein function. Brain Malformation Recommendation: Follow recommendations set forth by the SVI in conjunction with specifications added by the Brain Malformation Group for quality metrics and minimum validation controls required PMID: 31892348 (**Supplementary Document 1**)

RULES FOR COMBINING PATHOGENIC CRITERIA

The numerical system presented in the Tavtigian et. al. publication was utilized for determining classification (PMID: 32720330)

Path Supporting +1
Path Moderate +2
Path Strong +4
Path Very Strong +8
Benign Supporting -1
Benign Moderate -2
Benign Strong -4
Benign Very Strong -8

Ranges:
>10: P
6 to 9: LP
0 to 5: VUS
-6 to – 1: LB
<-6: B

Supplementals

Document 1. (PS3)

Functional Assay Validation
PMID: 31892348
(1) The 4 classes of assays (phosphorylation, DEPTOR binding, cell survivability, cell proliferation) are considered "broadly accepted historically" for these genes.
(2) Any publication within the spreadsheet can be used as evidence for a *supporting* level of evidence (PS3).
(3) Any paper must have validation controls (positive and negative) in order to be used as evidence for a level *above supporting*.

Positive validation controls are defined as variants classified as pathogenic/likely pathogenic (P/LP) independent of the PS3 criterion. Negative validation controls are defined as variants classified as benign/likely benign independent of the BS3 criterion

8-34 variants are required for *moderate* evidence.
35+ variants are required for *strong* evidence.

(4) For a publication to be used for any strength of evidence *above supporting*, it must also meet the minimum criteria below, depending on the type of evidence:

Supplemental Table 1

Evidence from Phosphorylation/Deptor Binding/Cell Survivability Assay:
(Assay Controls)
Basic Positive Control – WT necessary
Basic Negative Control – Empty vector or blank transfection can be used.
Biological Replicates – not necessary
Technical Replicates – yes, documented in at least triplicate (You can contact the researcher and ask if it is not specifically mentioned in the publication.)

Evidence of Cell Proliferation:
Basic Positive Control - WT necessary
Basic Negative Control – Empty vector or blank transfection can be used.
Biological Replicates – Necessary for animal studies (e.g., each mouse is a replicate, need at least 2)
Technical Replicates – Necessary, multiple samples measured from the same animal or experiment done in triplicate (at least 3)

Table 2.

Supporting (PS4_supporting)	Moderate (PS4_moderate)	Strong (PS4_strong)	Very strong(PS4_VS)
.5-1.49 points	1.5-3.49 points	3.5-15.99 points	>/=16 points

Table 3.

Feature	Proposed score
*Neuropathology confirmatory of a malformation of cortical development (e.g. focal cortical dysplasia, polymicrogyria)	1
*Neuroimaging appearance consistent with a malformation of cortical development (without neuropathology)	0.75
*Neuroimaging demonstrating at least one large cerebral hemisphere with cortical malformation(s)	1
*Macrocephaly (>=2 SD) and Developmental Delay or Intellectual disability with cortical malformation	1
*Macrocephaly(>=2 SD) and Developmental Delay or Intellectual disability without cortical malformations	0.75
*Clinical diagnosis of megalencephaly-polymicrogyria-polydactyly-hydrocephalus syndrome; (MPPH) or megalencephaly-capillary malformation-polymicrogyria syndrome; (MCAP)	1
****Cells from patient derived tissue show an aberrant cell overgrowth phenotype or increased phosphorylation	1
*Segmental overgrowth or vascular malformation of a limb or region of the body	0.75
***Presence of this variant in a tumor sample (databases such as COSMIC can be used)	0.25

**Be cautious when determining how many people with a variant have a phenotype to ensure the same individual is not counted more than once.*
Elements to review in order to evaluate the identity of an individual patient/case:
-location of the lesion (right vs. left)
-sex of the individual
-age of onset of epilepsy or other symptoms for the individual
-additional demographic or medical information if available
****Ensure that the variant of interest is not located in a normal tissue found in COSMIC. When counting tumor occurrences, each separate primary in the same individual or primaries in multiple different individuals counts*

as a separate occurrence. Alternatively, two tumors in one person can count as 2 primary tumors if the onsets are separated by 10 years (if biochemical evidence is not available). A maximum of 9 tumor samples can be counted for an individual variant keeping with the idea that tumor data cannot be utilized for any strength above supporting.
*****This criteria can only be used once for a given variant.*

Table 4.

Gene	*MTOR*	*AKT3*	*PIK3CA*	*PIK3R2*
Transcript	NM_004958.3	NM_005465.4	NM_006218.3	NM_005027.3
PMIDs	23322780, 27482884, 21210909	28969385	26637981, 24459181, 27631024	26860062
Domain	kinase domain AA: 1382-1982 g.11259424-11188148	pleckstrin homology domain AA: 5-109 g.244006460-243809297	Kinase Ras-binding domain AA: 173-292 g.178917642- 178921394	SH2, sequence homology 2 domain AA: 328-716 g.18273092-18280065
Domain	FKBP-rapamycin-binding (FRB) domain AA: 2015-2114 g.11187854-11187076	catalytic kinase domain AA: 151-388 g.243801023-243708899	kinase domains AA: 322-483 g.178921482- 178928263 AA: 797-1068 g.178942582-178952149	
Domain		C-terminal Protein Kinase AA: 425-475 g.243675707-243668566	adaptor binding domain (PI3K ABD) AA: 31-108 g.178916704- 178916937	

Table 3.
The prevalence of focal cortical dysplasia is unknown but it can be estimated.

Rational	**Mathematics**
1% of the population has epilepsy	1/100
of that 1/3 have medically refractory epilepsy	1/3
of that 1/3 will have surgery and be found to have a cortical dysplasia	1/3
Multiple genes are known to cause cortical dysplasia and the maximum contribution of a single gene is not more than 1/3	1/3
Calculated Allele Frequency	**1/2700 (0.037%)**